

IN THE SPECIFICATION:

The following is a marked-up version the Specification pursuant to revised 37 C.F.R. §1.121, with instructions and markings showing changes made herein to the Specification as filed. Underlining denotes added text while brackets denote deleted text.

On page 2, in the paragraph beginning at line 2, please enter the following amendment:

It is an object herein to provide a protease variant containing a substitution of an amino acid at one or more residue positions equivalent to residue positions selected from the group consisting of 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3).

On page 2, in the paragraph beginning at line 7, please enter the following amendment:

A protease variant is described comprising an amino acid sequence having a substitution at one or more residue positions equivalent to residue positions selected from the group consisting of 7, 23, 26, 28, 29, 30, 31, 47, 66, 69, 73, 82, 85, 88, 90, 92, 93, 105, 113, 139, 148, 149, 150, 151, 178, 200, 201, 231, 233, 267 and 273 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3). The protease variant of claim includes at least one improved property selected from improved a) wash performance and b) stability as compared to the wild type. In one embodiment, the protease to which these variants is compared is the wild-type GG36 (SEQ ID. NO.6). The improved stability can be improved thermostability.

On page 2, in the paragraph beginning at line 16, please enter the following amendment:

The protease variants can be selected from at least one position equivalent to 7N, 23A, 26S, 26T, 28C, 28G, 28S, 28T, 29G, 30A, 31A, 31I, 31T, 31V, 47D, 65M, 66D, 66E, 73G, 73T, 82R, 85D, 85G, 85S, 85L, 85V, 85Y, 88S, 90A, 90I, 90M, 92E, 92R, 93A, 93G, 93S, 93T, 105D, 105E, 105G, 105R, 113D, 139A, 148G, 149A, 149F, 149G, 149H, 149S, 149W, 150A, 150C, 150F, 150L, 151V, 178S, 178C, 178L, 201C, 231G, 231S, 233G, 233V, 267R, 267I, 273S of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3).

On page 2, in the paragraph beginning at line 24, please enter the following amendment:

The protease variant having improved wash performance at about 20 degrees centigrade, at a concentration of 0.5 to 1.0 ppm protease and at water hardness conditions of about 3 grains per gallon mixed Ca²⁺/Mg²⁺ hardness (Japanese wash conditions) comprises a substitution of at least one residue equivalent to 31, 47, 85, 90, 92, 105, 113, 148, 149, 151, 174, 200 and 201 of *Bacillus amyloliquefaciens* (SEQ ID NO:3). The substitutions are selected

from the group consisting of 31I, 31V, 47S, 47D, 85G, 90V, 92E, 105D, 105E, 113D, 148W, 151V, 174G, 174S, 200S and 201C.

On page 2, in the paragraph beginning at line 31 and continuing on page 3 to line 8, please enter the following amendments:

The protease variant can also have improved wash performance at about 40 degrees centigrade, at a protease concentration of 0.3-0.5 ppm protease and at water hardness conditions of about 15 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness. The protease variant of having improved wash performance under these conditions comprises a substitution at one or more positions equivalent to [to] 31, 69, 82, 148, 201, 203, 231, 233, 258, 267 and 270 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) These protease variants can comprise at least one substitution at one or more positions equivalent to 31, 69, 82, 148, 201, 231, 233 and 267 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) is selected from the group of 31I, 31V, 69G, 82R, 148G, 201S, 231V, 233G and 267R.

On page 3, in the paragraph beginning at line 10, please enter the following amendments:

In some embodiments, the protease variants have [The protease variant of claim 1, wherein said variant has] improved wash performance at about 10 degrees to about 30 degrees centigrade, at a concentration of 1.0 ppm protease and at water hardness conditions of about 6 grains per gallon mixed $\text{Ca}^{2+}/\text{Mg}^{2+}$ hardness (North American conditions). These protease variants comprise a substitution at one or more positions equivalent to 61, 66, 105, 203 and 258 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3). These at least one substitution at one or more positions equivalent to 61, 66, 105, 203, 216 and 258 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) can be selected from the group of 61E, 66D, 105D, 105E, 203D, 203E, 216E and 258E.

On page 4, in the paragraph beginning at line 1, please enter the following amendments:

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' (SEQ ID NO:3) and *Bacillus lentus* (wild-type) (SEQ ID NO:6).

On page 6, in the paragraph beginning at line 10, please enter the following amendment:

Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 5, 6, 7, 8, 12, 23, 24, 26, 28-31, 34, 38, 43, 47, 50, 52, 57, 63, 65, 66, 69, 70, 72, 73, 73, 82 - 85, 86, 88, 89, 90, 92, 93, 99, 103, 105, 113, 114, 116, 117, 119, 121, 125, 136, 138, 139, 142, 145, 147-151, 172, 174, 176, 177, 178, 179, 193, 196, 198, 199, 200, 201, 202, 203, 204, 206, 207, 218, 219, 220, 223, 228, 229,

231, 232, 233, 250, 252, 258, 263, 264, 266, 267, 270 and 273 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are identified herein.

On page 6, in the paragraph beginning at line 17 and continuing through line 4 of page 7, please enter the following amendments:

Specific substitutions of amino acids at one or more residue positions equivalent to A1E, A1D, A1R, A1K, W6R, G7N, Q12H, G23A, F24S, V26S, V26T, V28C, V28S, V28T, A29G, V30A, L31A, L31I, L31T, L31V, T38S, N43D, G47D, G47S, L50F, G52E, T57A, G65M, T66D, T66E, G69_, I72C, I72L, I72V, A73L, A73G, A73T, A73V, L82R, A85D, A85G, A85L, A85S, A85V, A85Y, P86D, A88S, E89G, L90A, L90I, L90M, L90V, A92E, A92R, V93A, V93G, V93I, V93S, V93T, S99G, S103C, S105D, S105E, S105G, S105R, W113D, A114C, A114G, A114S, A114T, N116D, N117S, M119A, M119C, M119F, M119G, M119S, M119T, M119V, H120R, Q121I, G127A, S128D, S128L, E136R, V139A, A142E, R145G, V147C, V147G, V147L, V147S, L148G, L148W, V149A, V149F, V149G, V149H, V149S, V149W, V150A, V150C, V150F, V150L, A151V, S156E, S156D, A169G, R170M, A172T, A174G, A174S, A174T, G178C, G178L, G178S, I198A, I198L, I198M, I198V, I198T, M199V, A200S, P201C, P201S, V203R, V203D, V203E, V203L, V203S, N204D, Q206R, S216D, N218S, S216E, S216R, A231G, A231S, A232C, A232G, A232I, A232L, A232M, A232N, A231V, A232T, A232V, A232S, L233G, L233V, I246M, I246V, R247C, N252S, S256G, T253D, T253E, T253K, T253R, G258D, G258E, G258K, G258R, Y263H, G264S, L267I, L267R, A270L, A270V, A273S, T260A in *Bacillus lentus* (SEQ ID NO:6; using BPN' numbering). Specific combinations of amino acids having at least the combinations V26S / N218S; G69 / Q12R; L90V / N204D; V93A / S103C; V93T / E136G; V139A / V150A; A142E / E89G; L148G / F24S; V149S / Q12H; V150A / T38S; V150C / N218S; A174G / N204D; A174S / G52E / A172T; G178C / N43D; I198M / V93I; I198V / V30A; A200S / N204D; P201S / L50F; P201S / T57A; A231G / M119V; A232I / A108V; A231V / Q206R; A232M / N116D; A232N / I16D; G264S / R145G; L267I / Y263H; L267R / S99G; L267R / N252S; A270V / E136R; and A172T / A174S / G52E in *Bacillus lentus* (SEQ ID NO:6; using BPN' numbering).

On page 7, in the paragraph beginning at line 5, please enter the following amendments:

Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 14, 31, 61, 82, 92, 203, 233, 253, 258, 267 and 270 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are identified herein as providing improved wash performance under European wash conditions. Specific substitutions

of amino acids at one or more residue positions corresponding to these positions are described in the Examples.

On page 7, in the paragraph beginning at line 11, please enter the following amendment:

Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 31, 47, 61, 66, 85, 86, 88, 92, 105, 113, 148, 149, 151, 201, 203, 216, 253, and 258 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are identified herein as providing improved wash performance under Japanese wash conditions.

On page 7, in the paragraph beginning at line 16, please enter the following amendment:

Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 1, 61, 66, 105, 203, 216 and 258 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are identified herein as providing improved wash performance under North American conditions.

On page 7, in the paragraph beginning at line 20, please enter the following amendment:

Specific substitutions of amino acids at one or more residue positions equivalent to residue positions selected from the group consisting of 7, 8, 23, 26, 28-31, 65, 70, 72, 73, 85, 86, 88, 90, 93, 114, 119, 147-150, 177, 178, 198, 203, 228, 231, 232, 246 and 273 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are identified herein as providing improved thermostability under European wash conditions.[.]

On page 7, in the paragraph beginning at line 25, please enter the following amendments:

These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1 (SEQ ID NO:2). The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. In a preferred embodiment of the present invention, the precursor protease is *Bacillus lentus* subtilisin and the substitutions are made at the equivalent amino acid residue positions in *B. lentus* corresponding to those listed above.

On page 8, in the paragraph beginning at line 3, please enter the following amendments:

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which

sequence is known. For example, Fig. 2 herein shows the conserved residues as between *B. amyloliquefaciens* subtilisin (SEQ ID NO:3) and *B. lentus* subtilisin (SEQ ID NO:6). After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 98%, 95%, 90%, 85%, 80%, 75%, 70%, 50% or at least 45% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained. Siezen et al. (1991) Protein Eng. **4(7)**:719-737 shows the alignment of a large number of serine proteases. Siezen et al. refer to the grouping as subtilases or subtilisin-like serine proteases.

On page 8, in the paragraph beginning at line 18, please enter the following amendments:

For example, in Fig. 3, the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* (SEQ ID NO:3), *Bacillus subtilis* (SEQ ID NO:4), *Bacillus licheniformis* (*carlsbergensis*) (SEQ ID NO:5) and *Bacillus lentus* (SEQ ID NO:6) are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

On page 16, in the paragraph beginning at line 13, please enter the following amendment:

In another aspect of the invention, it has been determined that modification at one or more residue positions, for example by substitution, insertion or deletion of an amino acid equivalent to residue positions selected from the group consisting of 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 86, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 203, 207, 219, 220, 223, 229, 233, 250, 258, 266, 267, 270 and 273 of *Bacillus amyloliquefaciens* subtilisin (SEQ ID NO:3) are important in improving the wash performance of the enzyme. The amino acids substituted, inserted or deleted contemplated by the inventors include, but are not limited to alanine (Ala or A), arginine (Arg or R), aspartic acid (Asp or D), asparagines (Asn or N), cysteine (Cys or C), glutamic acid (Glu or E), glutamine (Gln or Q), glycine (Gly or G), histidine (His or H), isoleucine (Iso or I), leucine (Leu or L), lysine (Lys

or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophane (Trp or W), tyrosine (Tyr or Y) and/or valine (Val or V).

On page 16, in the paragraph beginning at line 26 and continuing through line 4 of page 17, please enter the following amendment:

One aspect of the present invention includes a protease variant further comprising at least one additional replaced amino acid at one or more residue positions equivalent to residue positions or selected from the group consisting of 6, 9, 11-12, 19, 25, 37-38, 54-59 68, 71, 89, 111, 115, 120, 121-122, 140, 175, 180, 182, 186, 187, 191, 194, 195, 226 234-238, 241, 260-262, 265, 268, 75, 129, 131, 136, 159, 164, 165, 167, 170, 171, 194, 195, 27, 36, 57, 76, 97, 101, 104, 120, 123, 206, 218, 222, 224, 235, 274, 2, 3, 4, 10, 15, 17, 20, 40, 44, 51, 52, 60, 91, 108, 112, 133, 134, 143, 144, 145, 146, 173, 211, 212, 239, 240, 242, 243, 245, 252, 255, 257, 259, 263, 269, 183, 184, 185, 192, 209, 210, 18, 117, 137, and 244 of *Bacillus amyloliquefaciens* (SEQ ID NO:3). Specific residues contemplated by the inventors include those equivalent to : I122A, Y195E, M222A, M222S, Y167A, R170S, A194P, D36, N76D, H120D, G195E, and K235N of *Bacillus amyloliquefaciens*, which variant is derived from a *Bacillus subtilis*. Those skilled in the art will recognize the protease variants having these modifications can be made and are described in US Patents 5,741,694; 6,190,900; and 6,197,567, expressly incorporated by reference herein.

On page 17, in the paragraph beginning at line 5, please enter the following amendment:

Still another aspect of the present invention includes a protease variant further comprising at least one additional replaced amino acid at one or more equivalent residue positions from the group consisting of 12, 271, 204, 103, 136, 150, 89, 24, 38, 218, 52, 172, 43, 93, 30, 50, 57, 119, 108, 206, 16, 145, 263, 99, 252, 136, 32, 155, 104, 222, 166, 64, 33, 169, 189, 217, 157, 156, 152, 21, 22, 24, 36, 77, 87, 94, 95, 96, 110, 197, 204 107, 170, 171, 172, 213, 67, 135, 97, 126, 127, 128, 129, 214, 215, 50, 124, 123 or 274 of *Bacillus amyloliquefaciens* (SEQ ID NO:3). Specific residues contemplated by the inventors include: Y217L, K27R, V104Y, N123S, T274A, N76D, S103A, V104I, S101G, S103A, V104I, G159D, A232V, Q236H, Q245R, N248D, N252K M50, M124 and M222S . Additional specific residues contemplated by the inventors include those equivalent to : Q12R, E271G, N204D, S103C, E136G, V150A, E89G, F24S, T38S, N218S, G52E, A172T, N43D, V93I, V30A, L50F, T57A, M119V, A108V, Q206R, I16D, R145G, Y263H, S99G, N252S, Q136R of *Bacillus amyloliquefaciens* (SEQ ID NO:3). Protease variants, recombinant DNA encoding mutants at these positions and/or methods for making these modifications are described in US patent Nos.

RE 34,606; 5,972,682; 5,185,258; 5,310,675; 5,316,941; 5,801,038; 5,972,682, 5,955,340 and 5,700,676, expressly incorporated by reference herein. . In addition, these modifications can also be made using direct *Bacillus* transformation methods as described in Provisional Application Ser. No. 60/423,087 (filed November 1, 2002; Neelam Amin and Volker Schellenberger). In one embodiment, the modifications were performed using fusion PCR techniques (Teplyakov, AV, et al, Protein Eng., 1992 Jul 5(5):413-20). Provisional Application Serial Number [___/_____] 60/440,792, filed concurrently this date (Chris Leeflang, et al.)

On page 19, in the paragraph beginning at line 14, please enter the following amendment:

A large number of protease variants can be produced and purified using methods well known in the art. Mutations can be made in *Bacillus amyloliquefaciens* (BPN') subtilisin (SEQ ID NO:3) or *Bacillus lentus* GG36 subtilisin (SEQ ID NO:6). The variants can be selected from the following: 5, 7, 23, 26, 28-31, 34, 47, 63, 65, 66, 69, 70, 73, 82 - 85, 88, 90, 92, 93, 105, 113, 125, 138, 139, 148-151, 176, 178, 179, 193, 196, 200, 201, 202, 207, 219, 220, 223, 229, 233, 250, 266, 267 and 273.

On page 20, in the paragraph beginning at line 23, please enter the following amendment:

The GG36 codons of interest are numbered according to the BPN' numbering (listed in Figures 1 A – C and 3A-B; SEQ ID NOS:1, 2 and 3, 4, 5, and 6, respectively).

On page 21, in the paragraph beginning at line 13, please enter the following amendment:
Forward *Apal* primer:

GTGTGTGGGCCCATCAGTCTGACGACC (SEQ ID NO:7)

On page 21, in the paragraph beginning at line 15, please enter the following amendment:
Reverse *Apal* primer:

GTGTGTGGGCCCTATTCGGATATTGAG (SEQ ID NO:8)